Characterization of the Estrogen Receptor in Two Antiestrogen-resistant Cell Lines, LY2 and T47D

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ABSTRACT

Drug resistance occurs frequently during breast cancer treatment with antiestrogens. Since antiestrogen action is mediated by the estrogen receptor (ER), we have examined both the structural and functional properties of the ER present in two breast cancer cell lines, LY2 and T47D, which proliferate rapidly in the presence of antiestrogens. The ER function in LY2 cells was indistinguishable from that of the parental tamoxifen-sensitive MCF-7 cells as assessed by estrogen regulation of two endogenous genes and estrogen-regulated transcription in a transient transfection system. RNase protection assays, sensitive enough to detect single base pair mismatches, showed that the sequence of the coding region of ER of LY2 and T47D cells was wild type. Thus the ER appears to be normal in two independently isolated breast cancer cell lines whose growth is resistant to the inhibitory effect of antiestrogens. Moreover by conducting the cell proliferation studies in a phenol red-free medium, we have demonstrated that the antiestrogen resistance of LY2 and T47D cells corresponds in fact to an estrogen-independent growth.

INTRODUCTION

Antiestrogens are nonsteroidal compounds which bind to the ER. They are unable to elicit a receptor-mediated increase in transcription of several estrogen-induced genes. They can thus efficiently antagonize the action of estrogens and are promising therapeutic agents for hormone-dependent cancers, such as breast cancers (2). Although ER/PR level determinations have proved useful for the prediction of response to hormone therapy, there remain many tumors that possess estrogen receptors yet are resistant to the growth-suppressive effect of antihormone (3). Since antiestrogen action is mediated by the estrogen receptor (4), an alteration in the receptor could well render the cell resistant to antihormones. In fact steroid insensitivity has been described for the glucocorticoid receptor in some lymphomas (5), for the vitamin D receptor in hypocalcemic rickets (6), and for the androgen receptor in a case of androgen insensitivity syndrome (7). In each case it has been attributed to an alteration in the receptor. Changes such as a decrease in hormone binding and defect in DNA binding of the hormone-receptor complex have been reported in dexamethasone-resistant lymphoma cell lines (8, 9). A point mutation in the DNA binding domain of the vitamin D receptor has been associated with hypocalcemic rickets (6), and study of the androgen receptor gene in a family with complete androgen insensitivity has revealed a deletion of the steroid binding domain (7).

The MCF-7 cell line is derived from a pleural effusion of a human breast cancer (10) and expresses functional estrogen receptor. Following treatment with physiological concentrations of estradiol, in culture, these cells show a markedly increased proliferation rate. Moreover, the rate of transcription of several genes [PR, p52 (11, 12), cathespin D (M, 52,000, 13), ER (14, 15)] is regulated by estrogens and antineoplastic agents in these cells. They thus provide a good in vitro model system to study hormone-dependent breast cancers. On the other hand, establishing an in vitro model system for tamoxifen-resistant breast cancers has been more difficult and little is known about the mechanism of such a resistance. The LY2 cell line was established by stepwise selection of MCF-7 cells grown in a medium containing increasing concentrations of the antiestrogen LY117018 (16). The establishment of such an antiestrogen-resistant variant of the MCF-7 cell line provides an excellent opportunity to investigate whether a mutation in the receptor could explain the observed antiestrogen resistance. The T47D cell line (17, 18) is an independently established cell line whose growth rate is unaffected by the presence of antiestrogen. T47D cells contain ER, but the ability of these receptors to function entirely normally has been questioned by several investigators (15, 18). Thus, these two independently derived cell lines, LY2 and T47D, provide a means of investigating the possible mechanisms of tamoxifen resistance in human breast cancers.

We have studied here both the structural and functional properties of the ER in the LY2 cell line in comparison with MCF-7, the parental cell line. We have found that they are indistinguishable with respect to the induction of estrogen-regulated genes. We have compared the primary sequence of the coding region of the ER mRNA by means of an RNase protection analysis sensitive enough to detect single base pair mismatches and found also no difference. Likewise the coding sequence of the ER mRNA in the T47D cell line was indistinguishable from that of the MCF-7 ER. An important outcome of this investigation has been the demonstration that resistance to growth inhibition by tamoxifen reflects in fact the estrogen independence of these cell lines for growth.

MATERIALS AND METHODS

Cell Culture. HeLa and MCF-7 cells were maintained in DMEM supplemented with FCS (19). LY2 cells were maintained in DMEM supplemented with dextran-coated charcoal treated FCS and $1 \mu M$ tamoxifen (16). T47D cells were grown in RPMI 1640 medium supplemented with FCS (18). Seven days before being used for a hormone-dependent assay, MCF-7 and LY2 cells were transferred to phenol red-free (20) DMEM supplemented with dextran-coated charcoal treated FCS (18).

RNA Preparation and Northern Analysis. HeLa, MCF-7, LY2, and T47D cell total RNA were prepared as described by Groudine et al. (21). After electrophoretic separation on a formaldehyde-agarose gel, RNA was transferred to filtered membrane (Amersham) and hybridized with $^{32}P$-labeled nick-translated complementary DNA probes (10).
characteristics of MCF-7 cells and of the tamoxifen-resistant phenol red (16). However, phenol red subclone LY2, their proliferation rates were studied in the from 1.7 to 3.4 and 4.5 days, respectively. However, estrogen-gens and antiestrogens were similar to those observed for LY2 effect on their own on the growth of either cell line in the cell lines, but the effect was much more marked in the case of time of 8 days (Fig. 1/4). LY2 cells grown under similar condi- has since been shown to act as a weak agonist (20). We therefore performed our growth studies in phenol red-free medium. RNase digestion and analysis of the protected fragments were performed as described by Winter et al. (26).

RESULTS

Effects of Estrogen and Antiestrogens on the Rate of Prolif- eration of MCF-7 and LY2 Cells. To determine the growth characteristics of MCF-7 cells and of the tamoxifen-resistant subclone LY2, their proliferation rates were studied in the presence and absence of estrogen and antiestrogens. Previous growth experiments on LY2 cells were carried out in the presence of the pH indicator phenol red (16). However, phenol red has since been shown to act as a weak agonist (20). We therefore performed our growth studies in phenol red-free medium.

MCF-7 cells grown in phenol red-free medium had a doubling time of 8 days (Fig. 1A). LY2 cells grown under similar conditions divided much more rapidly with a doubling time of 2 days (Fig. 1B). 17β-Estradiol increased the proliferation rate of both cell lines, but the effect was much more marked in the case of MCF-7 cells. The proliferation rate of MCF-7 cells increased to 480% of control to give a doubling time of 1.7 days (Fig. 1A). By contrast, the proliferation rate of LY2 cells increased to only 150% to give a doubling time of 1.3 days (Fig. 1B).

Both antiestrogens OHT at 10 nM and ICI at 10 nM had little effect on their own on the growth of either cell line in the absence of estrogen (Fig. 1). The T47D cell responses to estrogens and antiestrogens were similar to those observed for LY2 cells (data not shown).

As expected, addition of either 10 nM OHT or 10 nM ICI increased the doubling time of estrogen-treated MCF-7 cells from 1.7 to 3.4 and 4.5 days, respectively. However, estrogen-treated LY2 cells with OHT or ICI addition continued to proliferate relatively rapidly (doubling time of 1.5 and 1.6 days with OHT and ICI, respectively) (Fig. 1). Since estrogen stim- ulated the proliferation rate of LY2 cells by only 50%, the inefficient antiestrogen action on these cells reflects in fact their almost complete estrogen independence for growth.

Thus LY2 cells differ from their parent cell line MCF-7 in being able to proliferate rapidly without added estrogen. More- over, the rate of LY2 proliferation in the absence of estrogen is comparable to that of MCF-7 grown in the presence of estrogen. These observations led us to investigate whether the observed estrogen-independent phenotype of the variant cell line LY2 resulted from an altered function of the receptor.

Induction of pS2 and Cathepsin D (M, 52,000) mRNA in MCF-7 and LY2 Cells by Estrogen and Antiestrogens. The rate of transcription of the pS2 (11, 12) and cathepsin D (M, 52,000 protein) (13) genes is regulated by estrogen/antiestrogen. We have studied the expression of these genes in both MCF-7 and LY2 cells to investigate the functional status of the ER in LY2 cells in comparison with MCF-7 cells. MCF-7 and LY2 cells grown in phenol red-free medium for 1 week were treated with either estradiol (10 nM), OHT (10 nM), or ICI (10 nM) for 3 days. The levels of pS2 and cathepsin D mRNA were deter- mined by standard northern blot analysis. In both cell lines, estradiol treatment resulted in an increase in the steady state levels of both pS2 and cathepsin D mRNA (Fig. 2, compare lanes 1 and 2; lanes 5 and 6), whereas no significant increase was observed following treatment with either antiestrogen (compare lanes 1, 3, and 4; lanes 5, 7, and 8; see Fig. 2, legend). 36B4 RNA was used as an internal control, since its level is not affected by estrogen/antiestrogen treatment (11). Thus the ER in the LY2 cell line mediates the transcriptional effects of both estradiol and antiestrogens in a manner very similar to that of the MCF-7 ER.

Activation of an Exogenous Estrogen Responsive Element by the Endogenous ER of MCF-7 and LY2 Cells. A transient transfection assay was used to further confirm that the transcrip- tional activation properties of the LY2 cell line were iden- tical to those of the ER of the parent MCF-7 cell line. The vitk-CAT plasmid reporter gene contains the well characterized estrogen-responsive element of the Xenopus vitellogenin A2 gene (27) linked to the promoter of the herpes simplex virus 1
Cell Lines using an RNase Protection Assay. Because the above tests for ER function did not reveal any differences with respect to the wild-type protein, we directly examined the coding sequence of LY2 ER mRNA by RNase protection assay, sensitive enough to detect single base pair mismatches (25, 26). Four overlapping antisense RNA probes were made and hybridized to total RNA extracted from MCF-7, LY2, or T47D cells (Fig. 4D and “Materials and Methods”). RNA extracted from the ER-negative HeLa cell line was used in all cases as a negative control (Fig. 4, lanes 3, 9, and 14). Probe A (Fig. 4A, lane 2) which corresponds to nucleotides 1-1020 of the ER mRNA-coding region plus 12 nucleotides of the 5′-untranslated sequence and 1358 bases of BSM vector sequences, resulted in a specific major protected fragment of 1 kilobase in the RNase protection assay using either MCF-7, LY2, or T47D cell RNA (Fig. 4A, lanes 4-6, arrow). This band was not detected by using HeLa cell RNA (Fig. 4A, lane 3). Probe B (Fig. 4B, lane 8) which corresponds to nucleotides 1035-1785 of the ER-coding sequence plus 15 nucleotides of 3′-untranslated sequence and 226 bases of BSM vector yielded a specific protected fragment of 783 bases on hybridization with MCF-7, LY2, or T47D cell RNA and subsequent RNase digestion (Fig. 4B, compare lanes 9-12, arrow). To ensure that a mismatch close to the ends of probes A and B was not being overlooked an overlapping probe covering nucleotides 480-1272 of the ER-coding sequence and 1355 nucleotides of vector sequence (probe C) (Fig. 4C, lane 13) was used. On hybridization with MCF-7, LY2, or T47D RNAs (Fig. 4C, lanes 15-17, arrow), probe C (2148 nucleotides) gave a protected fragment of the expected size (793 nucleotides) which was not present in the HeLa cell RNA control lane (Fig. 4C, lane 14). Additional bands, located above and below the specific bands, were also present in the HeLa cell RNA control lanes. They correspond most probably to either partial digestion products of the probes or of the specific protected fragments.

**DISCUSSION**

Estrogen Receptor Present in LY2 and T47D Cells has Wild-type Properties. LY2 is a breast cancer cell line resistant to the growth-inhibitory effects of antiestrogens despite containing estrogen receptors at levels comparable to those of the tamoxifen-sensitive parental MCF-7 cell line (approximately 500 fmol/mg DNA; data not shown). The properties of the ER of this cell line were characterized here to determine whether its mutation could be the basis for antiestrogen resistance. The transcription of two known estrogen-responsive genes, the pS2 and cathepsin D (M, 52,000) genes was studied as a test of receptor function. Estradiol administration caused an increase in both pS2 and cathepsin D mRNA in both LY2 and MCF-7 cell lines, whereas antiestrogens did not, consistent with observations made at the protein level by Davidson et al. (28) and Cavailles et al. (13). However we did not observe reproducibly the cathepsin D mRNA induction with OHT which was reported by one of these groups (13). Thus the estrogen-induced transcription of these genes is unchanged in LY2 cells, which suggests that ER function is not altered.

The receptor function was further examined in a transient transfection assay wherein the reporter gene vit-tk-CAT was introduced into LY2 cells. CAT activity was greatly stimulated by estradiol administration and this activity was inhibited by both antiestrogens OHT and ICI, as in the tamoxifen-sensitive parental MCF-7 cell line. These findings provide strong evidence for the presence of a functional ER in LY2 cells, whose transcriptional activating properties are unaltered when compared with those of MCF-7 ER. To further support this conclusion, we directly examined the structure of the coding sequence of the ER. RNase protection analysis which is sensitive enough to detect single base pair mismatches, confirmed that the LY2 ER-coding sequence was wild type. Similar analysis performed on cellular RNA from an independently isolated breast cancer cell line T47D, also resistant to the growth-inhibitory effect of antiestrasol.
certain mismatches, depending upon the surrounding bases may exist. They are identified using specific promoters as indicated and terminated at either the XmnI (probes A and C) or PvuII (probe B) cleavage sites.

Three domains of the ER-coding sequence (19) are diagrammed A to F with codon numbers given on top. The regions of the ER cDNA subcloned for the analysis are represented below (probes A, B, and C). The regions of ER-coding sequence encompassing the A/B, C, D, and part of the E domains (see also "Materials and Methods"). Antisense RNA probes were initiated at the indicated positions. The presence of mutations in these receptors is however unlikely, since both T47D and LY2 cell ERs bind estradiol with wild type characteristics (16, 18) and the transcriptional function of LY2 ER is apparently normal.

Antiestrogen Resistance Reflects Estrogen-independent Growth. Although no difference in ER structure or function was detected between the MCF-7 and LY2 cell lines, a striking difference in the "basal" rate of proliferation was found in the absence of estrogen. The parental MCF-7 cells divided very slowly in the absence of estrogen and their growth was strongly stimulated by estrogen. While the LY2 variant cell line proliferated almost optimally without estrogen. Furthermore, the "basal" rate of growth of MCF-7 and LY2 cells was not affected by the addition of antiestrogens OHT and ICI. This dramatic difference in the "basal" rates of proliferation of MCF-7 and LY2 cells was not noted in earlier studies (16), most probably because they were conducted in the presence of phenol red, a weak estrogen agonist, which in fact stimulates MCF-7 cell growth (20). Thus, in the work of Bronzert et al. (16), the antiestrogens OHT and LY117018, very likely blocked the effect of phenol red, resulting in a decreased rate of proliferation of MCF-7 cells in the absence of estrogen. It is clear from our present data that the "basal" rate of proliferation of both MCF-7 and LY2 cells are not affected by antiestrogen and that the higher "basal" rate of LY2 cell proliferation is only weakly increased by estrogens. Thus MCF-7 cells are estrogen-sensitive because of their estrogen requirement for proliferation. On the other hand, LY2 cells look antiestrogen resistant, because they are mostly estrogen independent for their growth, although their ER is functional.

Since LY2 ER structure and function is indistinguishable from that of MCF-7 ER by several criteria, the most complete estrogen independence of LY2 cells is not explicable by alterations in the estrogen receptor. Thus the control which is exerted by estrogen on the synthesis of some growth-stimulatory factors or growth inhibitors in the MCF-7 cell line are apparently no longer operating in the LY2 cell line. Estrogen treatment has been shown to enhance the secretion of several growth factors such as platelet-derived growth factor (30), IGF-I (31), and TGF-α (32) in the MCF-7 cell line. TGF-α can act through the EGF receptor (33). Moreover, PS2 and cathepsin D gene transcription is increased by estradiol and it has been suggested that they may function as growth factors (22, 34). One might expect that the regulation of one or more of these estrogen-regulated growth factors is altered in the LY2 cells in comparison with the MCF-7 cell line. The fact that LY2 cells continued to proliferate in the presence of antiestrogens, despite the observed inhibition of PS2 and cathepsin D gene expression, indicates that PS2 and cathepsin D gene products are not playing a key role in the antiestrogen-resistant growth of these cells, but does not exclude the possibility that they may be important for MCF-7 cell growth. Moreover, since PS2 gene transcription is increased by some growth factors including EGF (35), the observation that PS2 mRNA levels were not constitutively elevated in LY2 cells indicates that growth factors such as EGF or TGF-α are not constitutively overproduced by these cells. We note also that a recent report from Arteaga et al. (36) indicates that, although estrogen stimulates TGF-α secretion in MCF-7 cells, this increase in TGF-α is not a primary cause for enhanced proliferation in the presence of estradiol.

That selection of MCF-7 cells in a medium supplemented with the antiestrogen LY117018 resulted in variants that are estrogen-independent for growth, although their ER is functioning normally, is particularly relevant to cases of breast cancer that are classified as ER-positive, but are resistant to tamoxifen treatment or become so during the course of tamoxifen therapy. Note in this respect that the true antiestrogen ICI 164384 (37) is not more efficient at blocking the growth of LY2 cells than hydroxytamoxifen which is known to have both agonistic and antagonistic effects (38). Evidently, there has been a selection in the LY2 cells for an estrogen-independent growth-stimulatory machinery or the suppression of a growth-
inhibitory one. This could represent a general mechanism in the progression of breast cancer from tamoxifen sensitivity to insensitivity. Relevant to this, ras-transformed MCF-7 cells are also estrogen-independent for growth (39). These transformants display constitutively elevated expression of growth factors such as TGF-α and IGF-I, which appears to be under estrogen regulation in the parental cell line. It is also interesting to note that the regulation of the growth inhibitor TGF-β is altered in the LY2 cell line (40), inasmuch as it can no longer be induced by antiestrogen treatment of these cells in contrast to the MCF-7 parental cells.

Studies on the breast cancer cell line T47D have also revealed that tamoxifen resistance reflects an estrogen independence for growth (18). Previous reports have suggested that the ER may be altered in this cell line although the amounts of ER and estrogen responsiveness seem to vary in different clones of this cell line studied in different laboratories (15, 18). The cell line used in this investigation expresses ER protein at approximately 150 fmol/mg DNA (data not shown). The present results of RNase protection analysis indicate that the coding sequence of the ER mRNA of T47D cells studied is wild type (see also 25). That the ER in T47D cells is functionally wild type is further supported by the observation that the reporter gene vitk-CAT can be effectively activated by estradiol administration when transfected into these cells (refs. 27, 41, and references therein). Thus LY2 and T47D cells provide independent examples of estrogen-independent growth despite the presence of functional receptors.

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